TITLE OF THE INVENTION

ADENOVIRUS SEROTYPE 34 VECTORS, NUCLEIC ACIDS AND VIRUS PRODUCED THEREBY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. provisional application serial no. 60/458,825, filed on March 28, 2003.

BACKGROUND OF THE INVENTION

Adenoviruses are nonenveloped, icosahedral viruses that have been identified in several avian and mammalian hosts; Horne *et al.*, 1959 *J. Mol. Biol.* 1:84-86; Horwitz, 1990 In *Virology*, eds. B.N. Fields and D.M. Knipe, pps. 1679-1721. The first human adenoviruses (Ads) were isolated over four decades ago. Since then, over 100 distinct adenoviral serotypes have been isolated which infect various mammalian species, 51 of which are of human origin; Straus, 1984, In *The Adenoviruses*, ed. H. Ginsberg, pps. 451-498, New York:Plenus Press; Hierholzer *et al.*, 1988 *J. Infect. Dis.* 158:804-813; Schnurr and Dondero, 1993, *Intervirology*; 36:79-83; Jong *et al.*, 1999 *J Clin Microbiol.*, 37:3940-5. The human serotypes have been categorized into six subgenera (A-F) based on a number of biological, chemical, immunological and structural criteria which include hemagglutination properties of rat and rhesus monkey erythrocytes, DNA homology, restriction enzyme cleavage patterns, percentage G+C content and oncogenicity; Straus, *supra*; Horwitz, *supra.*

The adenovirus genome is very well characterized. It consists of a linear double-stranded DNA molecule of approximately 36,000 base pairs, and despite the existence of several distinct serotypes, there is some general conservation in the overall organization of the adenoviral genome with specific functions being similarly positioned.

Adenovirus has been a very attractive target for delivery of exogenous genes. The biology of adenoviruses is very well understood. Adenovirus has not been found to be associated with severe human pathology in immuno-competent individuals. The virus is extremely efficient in introducing its DNA into the host cell and is able to infect a wide variety of cells. Furthermore, the virus can be produced at high virus titers in large quantities. In addition, the virus can be rendered replication defective by deletion of the essential early-region 1 (El) of the viral genome; Brody et al, 1994 *Ann N Y Acad Sci.*, 716:90-101.

Replication-defective adenovirus vectors have been used extensively as gene transfer vectors for vaccine and gene therapy purposes. These vectors are propagated in cell

lines that provide E1 gene products *in trans*. Supplementation of the essential E1 gene products *in trans* is very effective when the vectors are from the same or a very similar serotype. E1-deleted group C serotypes (Ad1, Ad2, Ad5 and Ad6), for instance, grow well in 293 or PER.C6 cells which contain and express the Ad5 E1 region. However, the Ad5 E1 sequences in 293 or PER.C6 cells do not fully complement the replication of all serotypes other than group C. This is perhaps due to the inability of the Ad5 (group C) E1B 55K gene product to functionally interact with the E4 gene product(s) of the non-group C serotypes. Although the interaction is conserved within members of the same subgroup, it has not been found to be well conserved between subgroups. In order to successfully and efficiently rescue recombinant adenovirus of alternative, non-group C serotypes, a cell line expressing the E1 region of the serotype of interest would have to be generated. Alternatively, available Ad5E1-expressing cell lines could be modified to express Ad5E4 (or Orf6) in addition to Ad5E1. These additional, sometimes tedious and daunting tasks, impeded the production of recombinant, non-group C adenoviral vectors.

An efficient means for the propagation and rescue of alternative serotypes in an Ad5 E1-expressing cell line (such as PER.C6 or 293) was disclosed in pending U.S. provisional application (Serial No. 60/405,182, filed August 22, 2002). This method involves the incorporation of a critical E4 region into the adenovirus to be propagated. The critical E4 region is native to a virus of the same or highly similar serotype as that of the E1 gene product(s), particularly the E1B 55K region, of the complementing cell line, and comprises, in the least, nucleic acid encoding E4 Orf6.

Presently, two well-characterized adenovirus serotypes from subgroup C, Ad5 and Ad2, are the most widely used gene delivery vectors. There is a need to develop alternate Ad serotypes as gene transfer vectors since neutralizing antibodies in the general population may limit primary dosing or redosing with the same serotype. The prevalence of neutralizing antibody can vary from serotype to serotype. Neutralizing antibodies to some serotypes such as Ad5 are common, while antibodies to others are relatively rare. Alternate serotypes, furthermore, possess alternate tropisms which may lead to the elicitation of superior immune responses when used for vaccine or gene therapy purposes.

Adenovirus serotype 34, a subgroup B adenovirus, was originally isolated in 1972 and established as a recognized reference strain in 1975 (J.C. Hierholzer *et al.*, 1975 *J. Clin. Microbiol.* 1:366-376). Its antigenic relationship to 46 other human adenoviruses determined in reference horse antisera has been discussed; J.C. Hierholzer *et al.*, 1991 *Arch. Virol.* 121:179-197. Partial sequence information is available for Ad34. There have been several disclosures relating to Ad34 hexon sequences. The complete sequence of Ad34 hexon with some 5' and 3'

flanking sequence (3358 bp) was deposited in GenBank (Accession No. AB052911) by Mukouyama. A partial sequence of Ad34 hexon (1449 bp) was disclosed in Takeuchi *et al.*, 1999 *J. Clin. Microbiol.* 37:3392-3394, and GenBank (Accession No. AB018426). A partial sequence of Ad34 hexon (253 bp) was disclosed in Allard *et al.*, 2001 *J. Clin. Microbiol.* 39: 498-505 , and deposited in GenBank (Accession No. AF161573). Perera and Cardosa deposited two partial sequences of Ad34 hexon (571bp and 301 bp) with GenBank (Accession Nos. AJ272610 and AJ250786). Sequence for the Ad34 fiber gene was deposited by Arun, Mukouyama and Inada with GenBank (Accession No. AB073168). The sequence of the virus associated RNA region (VA RNA1 & 2) for Ad34 (162 bp) was disclosed by Kidd *et al.*, 1995 *Virology* 207:32-45, and GenBank (Accession No. U10677). Moreover, the sequence of the virus associated RNA region for Ad34 and partial sequence for the pre-terminal protein and 52/55K proteins (354 bp) was disclosed in Ma & Matthews, 1996 *J. Virol.* 70: 5083-99, and GenBank (Accession No. U52571). Adhikary, Mukouyama and Inada disclosed the sequence of the Ad34 genes for L4 100kDa, L4pVIII, E3 12.3kDa, E3 14.9kDa, E3 gp18.5kDa, E3 20.3kDa, E3 20.5kDa, E3 10.2kDa, E3 15.2kDa1, E3 15.2kDa2, and partial fiber sequence (4828 bp) and deposited the sequence with GenBank (Accession No. AB079724). The sequence of the right end of the viral genome (1038 bp) was disclosed in Chen & Horwitz, 1990 *Virology* 179:567-75, and GenBank (Accession No. M62712).

The fields of vaccines and gene therapy would greatly benefit from additional knowledge concerning alternative adenoviral serotypes, particularly those serotypes such as Ad34 which are not well represented in the human population. Of particular interest are recombinant adenoviral vectors based on alternative adenoviral serotypes, and means of obtaining such recombinant adenoviral vectors. This need in the art is met with the disclosure of the present application related to recombinant adenoviral vectors based on adenoviral serotype 34.

SUMMARY OF THE INVENTION

The present invention relates to recombinant, replication-deficient adenovirus vectors of serotype 34, a rare adenoviral serotype, and methods for generating the recombinant adenovirus based on the alternative serotype. Additionally, means of employing the recombinant adenovirus for the delivery and expression of exogenous genes are provided. The invention, thus, encompasses recombinant, replication-defective adenoviral vectors of serotype 34 which comprise one or more transgenes operatively linked to regulatory sequences which promote effective expression of the respective transgene(s). Host administration of such recombinant

adenovirus serotype 34 vectors, whether administered alone or in a combined modality and/or prime boost regimen, results in the efficient expression of the incorporated transgene and effectively induces an immune response capable of specifically recognizing the particular antigen administered (*e.g.*, HIV). Furthermore, the recombinant virus should evade pre-existing immunity to adenovirus serotypes which are more commonly encountered in the human population (*e.g.*, Ad5 and Ad2). The disclosed methods, thus, present an enhanced means for inducing an immune response against a particular antigen of interest (*e.g.*, HIV). Accordingly, the resultant immune response should offer a prophylactic advantage to previously uninfected individuals and/or provide a therapeutic effect by reducing viral load levels within an infected individual, thus prolonging the asymptomatic phase of infection.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the homologous recombination scheme utilized to recover pAd34ΔE1ΔE4Ad5Orf6.

Figure 2 illustrates the homologous recombination scheme utilized to recover pMRKAd34ΔE1ΔE4Ad5Orf6.

Figures 3A-1 to 3A-9 illustrate a nucleic acid sequence for wild-type adenovirus serotype 34 (SEQ ID NO: 1). The ATCC product number for Ad34 is VR-716.

Figure 4 illustrates the time course of SEAP expression using MRKAd5 and Ad34 vectors in rhesus macaques. Data represent cohort geometric means.

Figure 5 illustrates, in tabular format, T cell responses induced using MRKAd5 and Ad34 vectors expressing HIV-1 gag. Data are expressed in numbers of spot-forming cells per million PBMC (SFC/10^6 PBMC). "a" refers to a 20-mer peptide pool with 10-aa overlap and encompassing the entire HIV-1 CAM1 gag.

Figure 6 illustrates, in tabular format, the levels of CD4+ and CD8+ Gag-specific T cells in Ad34-immunized macaques at week 12. "a" refers to a 20-mer peptide pool with 10-aa overlap and encompassing the entire HIV-1 CAM1 gag.

Figure 7 illustrates the nucleic acid sequence (SEQ ID NO: 3) of the optimized human HIV-1 gag open reading frame.

Figure 8 illustrates the nucleic acid sequence encoding the gag expression cassette (SEQ ID NO: 4). The various regions of the figure are as follows: (1) a first underlined segment of nucleic acid sequence encoding the immediate early gene promoter region from human cytomegalovirus; (2) a first segment of lowercase letters which is not underlined, which segment of DNA contains a convenient restriction enzyme site; (3) a region in caps which contains the

coding sequence of HIV-1 gag; (4) a second segment of lowercase letters which is not underlined, which segment of DNA contains a convenient restriction enzyme site; and (5) a second underlined segment, this segment containing nucleic acid sequence encoding a bovine growth hormone polyadenylation signal sequence.

Figure 9 illustrates the nucleic acid sequence encoding the SEAP expression cassette (SEQ ID NO: 5). The various regions of the figure are as follows: (1) a first underlined segment of nucleic acid sequence encoding the immediate early gene promoter region from human cytomegalovirus; (2) a first segment of lowercase letters which is not underlined, which segment of DNA contains a convenient restriction enzyme site; (3) a region in caps which contains the coding sequence of the human placental SEAP gene; (4) a second segment of lowercase letters which is not underlined, which segment of DNA contains a convenient restriction enzyme site; and (5) a second underlined segment, this segment containing nucleic acid sequence encoding a bovine growth hormone polyadenylation signal sequence.

Figures 10A-1 to 10A-47 illustrate the nucleotide sequence of the pMRKAd5HIV-1gag vector (SEQ ID NO: 6 [coding] and SEQ ID NO: 7 [non-coding]).

Figures 11A-1 to 11A-10 illustrate a nucleic acid sequence for wild-type adenovirus serotype 35 (SEQ ID NO: 13). The ATCC product number for Ad35 is VR-718.

Figure 12 illustrates, in tabular format, T cell responses induced using a heterologous Ad34 prime/Ad35 boost regimen in macaques. "a" refers to a 20-mer peptide pool with 10-aa overlap and encompassing the entire HIV-1 CAM1 gag.

Figure 13 illustrates, in tabular format, the levels of CD4+ and CD8+ Gag-specific T cells in Ad34 primed/Ad35 boosted macaques at week 28. "a" refers to a 20-mer peptide pool with 10-aa overlap and encompassing the entire HIV-1 CAM1 gag.

DETAILED DESCRIPTION OF THE INVENTION

Rare adenoviral serotypes possess an inherent advantage over the more commonly exploited adenoviral serotypes (for instance, adenoviral serotypes 2 and 5) since preexisting immunity is unlikely to limit their efficient delivery and expression of exogenous genes to their target site. Different adenoviral serotypes also exhibit distinct tropisms by reason of their varying capsid structure and, thus, present the potential for targeting different tissues and possibly leading to the elicitation of superior immune responses when used for vaccine or gene therapy purposes. These rare adenoviral serotypes when rendered replication-defective, however, can be difficult to propagate and rescue in currently available adenoviral propagation cell lines.

Applicants have recently managed to successfully rescue and propagate one such rare, replication-defective alternative serotype, adenovirus serotype 34, a subgroup B adenovirus, and herein demonstrate the effective functioning of the adenovirus in the delivery and expression of exogenous transgenes.

Accordingly, the present invention relates to a recombinant adenoviral vector of serotype 34 suitable for use in gene therapy or vaccination protocols. The nucleic acid sequence for wild-type adenovirus serotype 34 (SEQ ID NO: 1) is illustrated in Figures 3A-1 to 3A-9, although any functional homologue or different strain of adenovirus serotype 34 can be utilized in accordance with the methods of the present invention, as one of ordinary skill in the art will appreciate. Ad34 sequence has been noted to differ in a few regions. The following sites are just a sampling of sequence variation that can be found in Ad34: (1) around base pair 10640 of SEQ ID NO: 1, a series of thirteen ("13") rather than twelve ("12") "T"s follow the sequence gtgagtccta (SEQ ID NO: 8); (2) around base pair 15372 of SEQ ID NO: 1, a series of fifteen ("15") or seventeen ("17") rather than sixteen ("16") "A"s follow the sequence ccgcactttct (SEQ ID NO: 9); (3) around base pair 17325 of SEQ ID NO: 1, a series of thirteen ("13") rather than twelve ("12") "A"s follow the sequence attgacattgg (SEQ ID NO: 10); and (4) around base pair 25717 of SEQ ID NO: 1, the sequence cagtctggagga (SEQ ID NO: 11) following the sequence ggagga (SEQ ID NO: 12) is deleted. Adenovirus serotypes have been distinguished through a number of art-appreciated biological, chemical, immunological and structural criteria which include hemagglutination properties of rat and rhesus monkey erythrocytes, DNA homology, restriction enzyme cleavage patterns, percentage G+C content and oncogenicity; Straus, *supra*; Horwitz, *supra*. A given serotype can be identified by a number of methods including restriction mapping of viral DNA; analyzing the mobility of viral DNA; analyzing the mobility of virion polypeptides on SDS-polyacrylamide gels following electrophoresis; comparison of sequence

information to known sequence particularly from capsid genes (*e.g.*, hexon) which contain sequences that define a serotype; and comparing a sequence with reference sera for a particular serotype available from the ATCC. Classification of adenovirus serotypes by SDS-PAGE has been discussed in Wadell *et al.*, 1980 *Ann. N.Y. Acad. Sci.* 354:16-42. Classification of adenovirus serotypes by restriction mapping has been discussed in Wadell *et al.*, *Current Topics in Microbiology and Immunology* 110:191-220. Adenovirus serotype 34, a subgroup B adenovirus, was originally isolated in 1972 and was established as a recognized reference strain in 1975 (J.C. Hierholzer *et al.*, 1975 *J. Clin. Microbiol.* 1:366-376). Its antigenic relationship to 46 other human adenoviruses determined in reference horse antisera has been discussed in the art; J.C. Hierholzer *et al.*, 1991 *Arch. Virol.* 121:179-197.

Adenovirus serotype 34 vectors in accordance with the present invention are at least partially deleted in E1 and devoid (or essentially devoid) of E1 activity, rendering the vector incapable of replication in the intended host. Preferably, the E1 region is completely deleted or inactivated. The adenoviruses may contain additional deletions in E3, and other early regions, albeit in situations where E2 and/or E4 is deleted, E2 and/or E4 complementing cell lines may be required to generate recombinant, replication-defective adenoviral vectors.

Adenoviral vectors of use in the methods of the present invention can be constructed using well known techniques, such as those reviewed in Hitt *et al.*, 1997 "Human Adenovirus Vectors for Gene Transfer into Mammalian Cells" *Advances in Pharmacology* 40:137-206, which is hereby incorporated by reference. Often, a plasmid or shuttle vector containing the heterologous nucleic acid of interest is generated which comprises sequence homologous to the specific adenovirus of interest. The shuttle vector and viral DNA or second plasmid containing the cloned viral DNA are then co-transfected into a host cell where homologous recombination occurs and results in the incorporation of the heterologous nucleic acid into the viral nucleic acid. Preferred shuttle vectors and cloned viral genomes contain adenoviral and plasmid portions. For shuttle vectors used in the construction of replication-defective vectors, the adenoviral portion typically contains non-functional or deleted E1 and E3 regions and the gene expression cassette, flanked by convenient restriction sites. The plasmid portion of the shuttle vector typically contains an antibiotic resistance marker under the transcriptional control of a prokaryotic promoter. Ampicillin resistance genes, neomycin resistance genes and other pharmaceutically acceptable antibiotic resistance markers may be used. To aid in the high level production of the nucleic acid by fermentation in prokaryotic organisms, it is advantageous for the shuttle vector to contain a prokaryotic origin of replication and be of high copy number. A number of commercially available prokaryotic cloning vectors

provide these benefits. Non-essential DNA sequences are, preferably removed. It is also preferable that the vectors not be able to replicate in eukaryotic cells. This minimizes the risk of integration of nucleic acid vaccine sequences into the recipients' genome. Tissue-specific promoters or enhancers may be used whenever it is desirable to limit expression of the nucleic acid to a particular tissue type.

Homologous recombination of the shuttle vector and wild-type adenovirus 34 viral DNA (Ad34 backbone vector) results in the generation of adenoviral pre-plasmids (see, for instance, pAd34ΔE1ΔE4Ad5Orf6, pMRKAd34ΔE1ΔE4Ad5Orf6, pAd34ΔE1gagΔE4Ad5Orf6, and pAd34ΔE1SEAPΔE4Ad5Orf6). Upon linearization, the pre-plasmids are capable of replication in PER.C6® cells or alternative E1-complementing cell lines. Infected cells and media can then be harvested once viral replication is complete.

A packaging cell will generally be needed in order to produce sufficient amount of adenovirus. The packaging cell should contain elements which are necessary for the production of the specific adenovirus of interest. It is preferable that the packaging cell and the vector not contain overlapping elements which could lead to replication competent virus by recombination. Specific examples of cells which are suitable for the propagation of recombinant Ad34 E1-deleted vectors express the early region 1 (E1) of adenovirus 34 or another group B serotype. Alternatively, propagation cell lines can be used which express adenoviral E1 and E4 regions (particularly, E4 open reading frame 6 ("ORF6")) which are derived from the same serotype but different subgroup than Ad34 (*e.g.*, Ad5 E1 and E4); *see, e.g.,* Abrahamsen *et al.*, 1997 *J. Virol.* 8946-8951, and U.S. Patent No. 5,849,561. Additionally, a cell line could be used that expresses E1B from Ad34 in addition to (1) E1A or (2) E1A and E1B from a serotype of a different subgroup. In copending U.S. provisional application serial no. 60/405,182, filed August 22, 2002, a strategy was disclosed for the efficient propagation and rescue of alternative adenoviral serotypes. The method is based on incorporating, into the genome of the adenovirus vector, an E4 region (or portion thereof including E4 ORF6) of the same or highly similar serotype as that of the E1 gene product(s), particularly E1B, being expressed by the complementing cell line. Examples 1-4 demonstrate the viability of such a method through the incorporation of an Ad5E4 region and its propagation in PER.C6 cells (which cells express Ad5E1). The wildtype adenovirus serotype 5 sequence is known and described in the art; *see* Chroboczek *et al.*, 1992 *J. Virol.* 186:280, which is hereby incorporated by reference. Placement of the E4 region or ORF6-containing portion is not critical. The critical step is making sure that either a promoter is supplied or the gene is strategically placed so that it runs off a promoter native to the vector (*e.g.*, such as the E4 promoter). The native E4 region of the vector can be

replaced, deleted or left intact. This method is, thus, suitable for use in the propagation and rescue of the adenoviral vectors of the present invention.

Typically, propagation cells are human cells derived from the retina or kidney, although any cell line capable of expressing the appropriate E1 and/or E4 region(s) can be utilized in the present invention. Embryonal cells such as amniocytes have been shown to be particularly suited for the generation of El complementing cell lines. Several cell lines are available. These include but are not limited to the known cell lines PER.C6 (ECACC deposit number 96022940), 911, 293, and El A549.

The present invention encompasses methods for producing a recombinant, replication-defective adenovirus of serotype 34 in an adenoviral E1-complementing cell line, comprising transfecting a recombinant, replication-defective adenoviral vector of serotype 34 in an adenoviral E1-complementing cell and allowing for the production of viral particles. The viral particles so produced form another aspect of the present invention. Host cells comprising the recombinant, replication-defective adenoviral serotype 34 vectors of the present invention form yet another aspect of the present invention; host cells being defined as a population of cells not including a transgenic human being. Recombinant, replication-defective adenovirus harvested in accordance with the methods of the present invention are encompassed herein as well. This harvested material may be purified, formulated and stored prior to host administration.

Adenoviral vectors in accordance with the present invention are very well suited to effectuate expression of desired proteins, especially in situations where an individual's immune response effectively prevents administration or readministration via the more commonly employed adenoviral serotypes. Accordingly, specific embodiments of the present invention are recombinant, replication-defective adenoviral vectors of serotype 34 which comprise a heterologous nucleic acid of interest. The nucleic acid of interest can be a gene, or a functional part of a gene. The nucleic acid can be DNA and/or RNA, can be double or single stranded, and can exist in the form of an expression cassette. The nucleic acid can be inserted in an E1 parallel (transcribed 5' to 3') or anti-parallel (transcribed in a 3' to 5' direction relative to the vector backbone) orientation. The nucleic acid can be codon-optimized for expression in the desired host (*e.g.*, a mammalian host). The heterologous nucleic acid can be in the form of an expression cassette. A gene expression cassette will typically contain (a) nucleic acid encoding a protein or antigen of interest; (b) a heterologous promoter operatively linked to the nucleic acid encoding the protein; and (c) a transcription termination signal.

In specific embodiments, the heterologous promoter is recognized by an eukaryotic RNA polymerase. One example of a promoter suitable for use in the present invention is the immediate early human cytomegalovirus promoter (Chapman *et al.*, 1991 *Nucl. Acids Res.* 19:3979-3986). Further examples of promoters that can be used in the present invention are the strong immunoglobulin promoter, the EF1 alpha promoter, the murine CMV promoter, the Rous Sarcoma Virus promoter, the SV40 early/late promoters and the beta actin promoter, albeit those of skill in the art can appreciate that any promoter capable of effecting expression in the intended host can be used in accordance with the methods of the present invention. The promoter may comprise a regulatable sequence such as the Tet operator sequence. Sequences such as these that offer the potential for regulation of transcription and expression are useful in instances where repression of gene transcription is sought. The adenoviral gene expression cassette may comprise a transcription termination sequence; specific embodiments of which are the bovine growth hormone termination/polyadenylation signal (bGHpA) or the short synthetic polyA signal (SPA) of 50 nucleotides in length defined as follows: AATAAAAGATCTTTATTTTCATTAGATCTGTGTGTT-GGTTTTTTGTGTG (SEQ ID NO:2). A leader or signal peptide may also be incorporated into the transgene. In specific embodiments, the leader is derived from the tissue-specific plasminogen activator protein, tPA.

Heterologous nucleic acids of interest are genes (or their functional counterparts) which encode immunogenic and/or therapeutic proteins. Preferred therapeutic proteins are those which elicit some measurable therapeutic benefit in the individual host upon administration. Preferred immunogenic proteins are any proteins which are capable of eliciting an immune response in an individual. Applicants have exemplified the delivery of a representative immunogenic protein (HIV gag) in the present specification in non-human primates (rhesus macaques), albeit any gene encoding a therapeutic or immunogenic protein can be used in accordance with the methods disclosed herein. The adenovirus serotype 34 vectors were found to induce significant levels of gag-specific T cells; Figure 5. Moreover, the results indicated that immunization with the disclosed vectors was able to elicit both HIV-specific CD4+ and CD8+ T cells; Figure 6.

An aspect of the present invention, therefore, relates to adenovirus serotype 34-based vectors carrying an HIV transgene. In these embodiments, nucleic acid encoding any HIV antigen may be utilized (specific examples of which include gag, pol, nef, gp160, gp41, gp120, tat, and rev, including derivatives of the aforementioned genes). The embodiments exemplified herein employ nucleic acid encoding a codon-optimized p55 gag antigen; *see* Figure 7 (SEQ ID NO: 3). Codon-optimized HIV-1 env genes are disclosed in PCT International Applications

PCT/US97/02294 and PCT/US97/10517, published August 28, 1997 (WO 97/31115) and December 24, 1997, respectively. Codon-optimized HIV-1 pol genes are disclosed in U.S. Application Serial No. 09/745,221, filed December 21, 2000 and PCT International Application PCT/US00/34724, also filed December 21, 2000. Codon-optimized HIV-1 nef genes are disclosed in U.S. Application Serial No. 09/738,782, filed December 15, 2000 and PCT International Application PCT/US00/34162, also filed December 15, 2000.

In this specific embodiment of a recombinant, replication-defective Ad34 vector comprising an HIV-1 gene, the gene may be derived from HIV-1 strain CAM-1; Myers et al, eds. "Human Retroviruses and AIDS: 1995, IIA3-IIA19, which is hereby incorporated by reference. This gene closely resembles the consensus amino acid sequence for the clade B (North American/European) sequence. HIV gene sequence(s) may be based on various clades of HIV-1; specific examples of which are Clades B and C. Sequences for genes of many HIV strains are publicly available from GenBank and primary, field isolates of HIV are available from the National Institute of Allergy and Infectious Diseases (NIAID) which has contracted with Quality Biological (Gaithersburg, MD) to make these strains available. Strains are also available from the World Health Organization (WHO), Geneva Switzerland. It is well within the purview of the skilled artisan to choose an appropriate nucleotide sequence which encodes a specific HIV antigen, or immunologically relevant portion or modification thereof. "Immunologically relevant" as defined herein means (1) with regard to a viral antigen, that the protein is capable, upon administration, of eliciting a measurable immune response within an individual sufficient to retard the propagation and/or spread of the virus and/or to reduce the viral load present within the individual; or (2) with regards to a nucleotide sequence, that the sequence is capable of encoding for a protein capable of the above.

The present invention encompasses methods for (1) effectuating a therapeutic response in an individual and (2) generating an immune response (including a cellular-mediated immune response) comprising administering to an individual a recombinant adenovirus serotype 34 vector in accordance with the present invention. One aspect of the present invention are methods for generating an enhanced immune response against one or more antigens (bacterial, viral (*e.g.*, HIV), or other (*e.g.*, cancer)) which comprise the administration of a recombinant adenovirus serotype 34 vehicle expressing the antigen of interest. Administration of recombinant Ad34 vectors in this manner provides for improved cellular-mediated immune responses, particularly where there is pre-existing immunity in a given host to the more well-represented adenovirus serotypes (*e.g.*, Ad2 and Ad5). An effect of the improved vaccine administration methods should be a lower transmission rate to (or occurrence rate in) previously

uninfected individuals (*i.e.,* prophylactic applications) and/or a reduction in the levels of virus/bacteria/foreign agent within an infected individual (*i.e.,* therapeutic applications). As relates to HIV indications, an effect of the improved vaccine administration methods should be a lower transmission rate to previously uninfected individuals (*i.e.,* prophylactic applications) and/or a reduction in the levels of viral loads within an infected individual (*i.e.,* therapeutic applications) so as to prolong the asymptomatic phase of HIV infection. Administration, intracellular delivery and expression of the recombinant Ad34 vectors elicits a host CTL and Th response.

Accordingly, the present invention relates to methodology regarding administration of the recombinant Ad34 viral vectors (or immunogenic compositions thereof, herein termed vaccines) to provide effective immunoprophylaxis, to prevent establishment of an infection following exposure to the viral (for instance, HIV), bacterial or other agent, or as a post-infection therapeutic vaccine to mitigate infection to result in the establishment of a lower virus/bacteria/other load with beneficial long term consequences.

The recombinant adenovirus serotype 34 vectors of the present invention may be administered alone, or as part of a prime/boost administration regimen. A priming dose(s) of at least one antigen (*e.g.,* an HIV antigen) is first delivered with a recombinant adenoviral vector. This dose effectively primes the immune response so that, upon subsequent identification of the antigen(s) in the circulating immune system, the immune response is capable of immediately recognizing and responding to the antigen(s) within the host. The priming dose(s) is then followed with a boosting dose comprising a recombinant adenoviral vector containing at least one gene encoding the antigen. A mixed modality prime and boost inoculation scheme will result in an enhanced immune response, particularly where there is pre-existing anti-vector immunity. Prime-boost administrations typically involve priming the subject (by viral vector, plasmid, protein, *etc.*) at least one time, allowing a predetermined length of time to pass, and then boosting (by viral vector, plasmid, protein, *etc.*). Multiple primings, typically 1-4, are usually employed, although more may be used. The length of time between priming and boost may typically vary from about four months to a year, albeit other time frames may be used as one of ordinary skill in the art will appreciate.

In addition to a single protein or antigen of interest being delivered by the recombinant, replication-defective adenovirus serotype 34 vectors of the present invention, two or more proteins or antigens can be delivered either via separate vehicles or delivered *via* the same vehicle. Multiple genes/functional equivalents may be ligated into a proper shuttle plasmid for generation of a pre-adenoviral plasmid comprising multiple open reading frames. Open

reading frames for the multiple genes/functional equivalents can be operatively linked to distinct promoters and transcription termination sequences. In other embodiments, the open reading frames may be operatively linked to a single promoter, with the open reading frames operatively linked by an internal ribosome entry sequence (IRES; as disclosed in WO 95/24485), or suitable alternative allowing for transcription of the multiple open reading frames to run off of a single promoter. In certain embodiments, the open reading frames may be fused together by stepwise PCR or suitable alternative methodology for fusing together two open reading frames. Due consideration must be given, however, to the effective packaging limitations of the viral vehicle. Adenovirus type 5, for instance, has been shown to exhibit an upper cloning capacity limit of approximately 105% of the wildtype Ad5 sequence.

Prime-boost regimens can employ different adenoviral serotypes. One example of such a protocol would be a priming dose(s) comprising a recombinant adenoviral vector of a first serotype followed by a boosting dose comprising a recombinant adenoviral vector of a second and different serotype; *see,* for instance, Example 6 and Figures 12 and 13. Therein, a cohort of monkeys was given two doses of an Ad34-based HIV gag vector at weeks 0 and 4, and boosted at week 24 with an Ad35-based HIV gag vector. Administration of the Ad35-based vector resulted in about a 3-fold enhancement in T cell responses when compared to the levels at the time of the booster. In an alternative embodiment, the priming dose can comprise a mixture of separate adenoviral vehicles each comprising a gene encoding for a different protein/antigen. In such a case, the boosting dose would also comprise a mixture of vectors each comprising a gene encoding for a separate protein/antigen, provided that the boosting dose(s) administers recombinant viral vectors comprising genetic material encoding for the same or similar set of antigens that were delivered in the priming dose(s). These multiple gene/vector administration modalities can further be combined. It is further within the scope of the present invention to embark on combined modality regimes which include multiple but distinct components from a specific antigen.

Compositions, including vaccine compositions, comprising the adenoviral vectors of the present invention are an important aspect of the present invention. These compositions can be administered to mammalian hosts, preferably human hosts, in either a prophylactic or therapeutic setting. Potential hosts/vaccinees include but are not limited to primates and especially humans and non-human primates, and include any non-human mammal of commercial or domestic veterinary importance. Compositions comprising recombinant adenoviral serotype 34 vectors may be administered alone or in combination with other viral- or non-viral-based DNA/protein vaccines. They also may be administered as part of a broader treatment regimen.

The present invention encompasses those situations as well where the disclosed recombinant adenoviral serotype 34 vectors are administered in conjunction with other therapies; for example, HAART therapy (in the case of a recombinant HIV vector).

Compositions comprising the recombinant viral vectors may contain physiologically acceptable components, such as buffer, normal saline or phosphate buffered saline, sucrose, other salts and polysorbate. In certain embodiments, the formulation has: 2.5-10 mM TRIS buffer, preferably about 5 mM TRIS buffer; 25-100 mM NaCl, preferably about 75 mM NaCl; 2.5-10% sucrose, preferably about 5% sucrose; 0.01 -2 mM $MgCl_2$; and 0.001%-0.01% polysorbate 80 (plant derived). The pH should range from about 7.0-9.0, preferably about 8.0. One skilled in the art will appreciate that other conventional vaccine excipients may also be used in the formulation. In specific embodiments, the formulation contains 5mM TRIS, 75 mM NaCl, 5% sucrose, 1mM $MgCl_2$, 0.005% polysorbate 80 at pH 8.0. This has a pH and divalent cation composition which is near the optimum for Ad5 and Ad6 stability and minimizes the potential for adsorption of virus to a glass surface. It does not cause tissue irritation upon intramuscular injection. It is preferably frozen until use.

The amount of viral particles in the vaccine composition to be introduced into a vaccine recipient will depend on the strength of the transcriptional and translational promoters used and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of $1x10^7$ to $1x10^{12}$ particles and preferably about $1x10^{10}$ to $1x10^{11}$ particles is administered directly into muscle tissue. Subcutaneous injection, intradermal introduction, impression through the skin, and other modes of administration such as intraperitoneal, intravenous, or inhalation delivery are also contemplated. Parenteral administration, such as intravenous, intramuscular, subcutaneous or other means of administration of interleukin-12 protein, concurrently with or subsequent to parenteral introduction of the vaccine compositions of this invention is also advantageous.

The following non-limiting Examples are presented to better illustrate the workings of the invention.

Example 1

Construction of pAd34ΔE1ΔE4Ad5Orf6

To generate an E1- Ad34 based vector that can propagate in existing group C/Ad5 E1 complementing cell lines (293, PER.C6), Ad5 Orf6 was inserted in place of the native E4 region. To construct the Ad34 pre-Adenovirus plasmid, advantage was taken of the sequence

homology between Ad34 and Ad35. Cotransformation of BJ 5183 bacteria with purified wild-type Ad34 viral DNA and the appropriately constructed Ad35 ITR cassette resulted in the circularization of the viral genome by homologous recombination. The construction of the pre-Ad plasmid based on Ad34, is outlined below:

To construct pAd34ΔE1ΔE4Ad5Orf6 (An Ad34 pre-Ad plasmid containing an E1 deletion and an E4 deletion substituted with Ad5 Orf6), we utilized an Ad35 ITR cassette. We anticipated that sequence homology between Ad34 and Ad35 would allow homologous recombination to occur. The Ad35 ITR cassette was constructed containing sequences from the right (bp 31599 to 31913 and bp 34419 to 34793) and left (bp 4 to 456 and bp 3403 to 3886) end of the Ad35 genome (*see* Figures 11A-1 to 11A-10) separated by plasmid sequences containing a bacterial origin of replication and an ampicillin resistance gene. The four segments were generated by PCR and cloned sequentially into pNEB193, generating pNEBAd35-4. Next the Ad5 Orf6 open reading frame was generated by PCR and cloned between Ad35 bp 31913 and 34419 generating pNEBAd35-4Ad5Orf6 (the ITR cassette). PNEB193 is a commonly used commercially available cloning plasmid (New England Biolabs cat# N3051S) containing a bacterial origin of replication, ampicillin resistance gene and a multiple cloning site into which the PCR products were introduced. The ITR cassette contains a deletion of E1 sequences from Ad35 bp 457 to 3402 with a unique *Swa* I restriction site located in the deletion and an E4 deletion from Ad35 bp 31914 to 34418 into which Ad5 Orf6 was introduced in an E4 parallel orientation. In this construct Ad5Orf6 expression is driven by the Ad35 E4 promoter. The Ad35 sequences (bp 31599 to 31913 and bp 3403 to 3886) in the ITR cassette provided regions of homology with the purified Ad34 viral DNA in which bacterial recombination could occur following cotransformation into BJ 5183 bacteria (Figure 1). The ITR cassette was also designed to contain unique restriction enzyme sites (*Pme*I) located at the end of the viral ITR's so that digestion would release the recombinant Ad34 genome from the plasmid sequences. Potential clones were screened by restriction analysis and one clone was selected as pAd34ΔE1ΔE4Ad5Orf6.

Example 2

Rescue of pAd34ΔE1ΔE4Ad5Orf6 into Virus

In order to determine if pre-adenovirus plasmid pAd34ΔE1ΔE4Ad5Orf6, could be rescued into virus and propagated in a group C E1 complementing cell line, the plasmid was digested with *Pme* I and transfected into T-25 flasks of PER.C6 cells using the calcium phosphate co-precipitation technique (Cell Phect Transfection Kit, Amersham Pharmacia

Biotech Inc). *Pme*I digestion releases the viral genome from plasmid sequences allowing viral replication to occur after cell entry. Viral cytopathic effect (CPE), indicating that virus replication and amplification was occurring was observed following transfection. When CPE was complete, approximately 7-10 days post transfection, the infected cells and media were harvested, freeze/thawed three times and the cell debris pelleted by centrifugation. Approximately 1 ml of the cell lysate was used to infect a T-225 flask of PER.C6 cells at 80-90% confluence. Once CPE was reached, infected cells and media were harvested, freeze/thawed three times and the cell debris pelleted by centrifugation. Clarified cell lysates were then used to infect 2-layer NUNC cell factories of PER.C6 cells. Following complete CPE, the virus was purified by ultracentrifugation on CsCl density gradients. In order to verify the genetic structure of the rescued viruses, viral DNA was extracted using pronase treatment followed by phenol chloroform extraction and ethanol precipitation. Viral DNA was then digested with *Hind*III and treated with Klenow fragment to end-label the restriction fragments with P33-dATP. The end-labeled restriction fragments were then size-fractionated by gel electrophoresis and visualized by autoradiography. The digestion products were compared with the digestion products of the corresponding pre-Adenovirus plasmid (that had been digested with *Pme*1/*Hind*III prior to labeling) from which they were derived. The expected sizes were observed, indicating that the viruses had been successfully rescued.

Example 3

Insertion of an Expression Cassette into pAd34ΔE1ΔE4Ad5Orf6

In order to introduce a gag or SEAP expression cassette (*see* Figures 8 and 9, respectively) into the E1 region of pAd34ΔE1ΔE4Ad5Orf6, bacterial recombination was again used. A gag expression cassette consisting of the following: 1) the immediate early gene promoter from human cytomegalovirus, 2) the coding sequence of the human immunodeficiency virus type 1 (HIV-1) gag (strain CAM-1; 1526 bp) gene, and 3) the bovine growth hormone polyadenylation signal sequence, was cloned into the E1 deletion in Ad35 shuttle plasmid, pNEBAd35-2 (a precursor to the Ad35 ITR cassettes described above), generating pNEBAd35CMVgagBGHpA. pNEBAd35-2 contains Ad35 sequences from the left end of the genome (bp 4 to 456 and bp 3403 to 3886) with a unique *Swa*I site between bp 456 and 3403 at the position of the deletion. The gag expression cassette was obtained from a previously constructed shuttle plasmid by *Eco*RI digestion. Following the digestion the desired fragment was gel purified, treated with Klenow to obtain blunt ends and cloned into the *Swa*I site in pNEBAd35-2. This cloning step resulted in the gag expression cassette being inserted into the

E1 deletion between bp 456 and 3403 in the E1 parallel orientation. The shuttle vector containing the gag transgene was digested to generate a DNA fragment consisting of the gag expression cassette flanked by Ad35 bp 4 to 456 and bp 3403 to 3886 and the fragment was purified after electrophoresis on an agarose gel. Cotransformation of BJ 5183 bacteria with the shuttle vector fragment and pAd34ΔE1ΔE4Ad5Orf6, linearized in the E1 region by digestion with *Swa* I, resulted in the generation of the Ad34 gag-containing pre-Adenovirus plasmid pAd34ΔE1gagΔE4Ad5Orf6 by homologous recombination. Potential clones were screened by restriction analysis.

A similar strategy was used to generate Ad34 pre-Ad plasmids containing a SEAP expression cassette. In this case a SEAP expression cassette consisting of: 1) the immediate early gene promoter from human cytomegalovirus, 2) the coding sequence of the human placental SEAP gene, and 3) the bovine growth hormone polyadenylation signal sequence was cloned into the E1 deletion in Ad35 shuttle plasmid, pNEBAd35-2, generating pNEBAd35CMVSEAPBGHpA. The SEAP expression cassette was obtained from a previously constructed shuttle plasmid by *Eco*RI digestion. Following the digestion the desired fragment was gel purified, treated with Klenow to obtain blunt ends and cloned into the *Swa*I site in pNEBAd35-2. The transgene was then recombined into the pAd34ΔE1ΔE4Ad5Orf6, generating pAd34ΔE1SEAPΔE4Ad5Orf6 as described above for the gag transgene.

All pre-Ad plasmids were rescued into virus and expanded to prepare CsCl purified stocks as described above.

Example 4

Construction of pMRKAd34ΔE1ΔE4Ad5Orf6

To construct an Ad34 pre-Ad plasmid that was composed entirely of Ad34 sequences, an Ad34 ITR cassette was generated. The Ad34 ITR cassette was constructed containing sequences from the right (bp 31584 to 31895 and bp 34409 to 34772) and left (bp 4 to 456 and bp 3402 to 3885) end of the Ad34 genome (*see* Figures 3A-1 to 3A-9) separated by plasmid sequences containing a bacterial origin of replication and an ampicillin resistance gene. These four segments were generated by PCR and cloned sequentially into pNEB193, generating pNEBAd34-4. Next the Ad5 Orf6 open reading frame was generated by PCR and cloned between Ad34 bp 31895 and 34409 generating pNEBAd34-4Ad5Orf6 (the ITR cassette). PNEB193 is a commonly used commercially available cloning plasmid (New England Biolabs cat# N3051S) containing a bacterial origin of replication, ampicillin resistance gene and a multiple cloning site into which the PCR products were introduced. The ITR cassette contains a

deletion of E1 sequences from Ad34 bp 457 to 3401 with a unique *Swa* I restriction site located in the deletion and an E4 deletion from Ad34 bp 31896 to 34408 into which Ad5 Orf6 was introduced in an E4 parallel orientation. In this construct Ad5Orf6 expression is driven by the Ad34 E4 promoter. The Ad34 sequences (bp 31584 to 31895 and bp 3402 to 3885) in the ITR cassette provided regions of homology with the purified Ad34 viral DNA in which bacterial recombination could occur following cotransformation into BJ 5183 bacteria (Figure 2). The ITR cassette was also designed to contain unique restriction enzyme sites (PmeI) located at the end of the viral ITR's so that digestion would release the recombinant Ad34 genome from the plasmid sequences. Potential clones were screened by restriction analysis and one clone was selected as pMRKAd34ΔE1ΔE4Ad5Orf6.

Example 5

In Vivo Studies

A. Immunization

Cohorts of 3 rhesus macaques were given single intramuscular injections of one of the two vectors: (1) 10^11 vp MRKAd5-SEAP (in MRKAd vector backbone of Figures 10A-1 to 10A-45 disclosed in PCT/US01/28861, published March 21, 2002); and (2) 10^11 vp Ad34ΔE1SEAPΔE4Ad5Orf6. Rhesus macaques were between 3-10 kg in weight. In all cases, the total dose of each vaccine was suspended in 1 mL of buffer. The macaques were anesthetized (ketamine/xylazine) and the vaccines were delivered i.m. in 0.5-mL aliquots into both deltoid muscles using tuberculin syringes (Becton-Dickinson, Franklin Lakes, NJ). Peripheral blood mononuclear cells (PBMC) were prepared from blood samples collected at several time points during the immunization regimen. All animal care and treatment were in accordance with standards approved by the Institutional Animal Care and Use Committee according to the principles set forth in the *Guide for Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council.

B. SEAP Assay

Serum samples were analyzed for circulating human secreted alkaline phosphatase (SEAP) levels using TROPIX phospha-light chemiluminescent kit (Applied Biosystems Inc). Duplicate 5 μL aliquots of each serum were mixed with 45 μL of kit-supplied dilution buffer in a 96-well white DYNEX plate. Serially diluted solutions of a human placental alkaline phosphatase (Catalog no. M5905, Sigma, St. Louis, MO) in 10% naïve monkey serum served to provide the standard curve. Endogenous SEAP activity in the samples was inactivated by heating the well for 30 minutes at 65 °C. Enzymatic SEAP activities in the samples were

determined following the procedures described in the kit. Chemiluminescence readings (in relative light units) were recorded using DYNEX luminometer. RLU readings were converted to ng/mL SEAP using a log-log regression analyses.

C. ELISPOT Assay

The IFN-γ ELISPOT assays for rhesus macaques were conducted following a previously described protocol (Allen *et al.*, 2001 *J. Virol.* 75(2):738-749), with some modifications. For antigen-specific stimulation, a peptide pool was prepared from 20-aa peptides that encompass the entire HIV-1 gag sequence with 10-aa overlaps (Synpep Corp., Dublin, CA). To each well, 50 μL of 2-4 x 10^5 peripheral blood mononuclear cells (PBMCs) were added; the cells were counted using Beckman Coulter Z2 particle analyzer with a lower size cut-off set at 80 femtoliters ("fL"). Either 50 μL of media or the gag peptide pool at 8 μg/mL concentration per peptide were added to the PBMC. The samples were incubated at 37°C, 5% CO_2 for 20-24 hrs. Spots were developed accordingly and the plates were processed using custom-built imager and automatic counting subroutine based on the ImagePro platform (Silver Spring, MD); the counts were normalized to 10^6 cell input.

D. Intracellular Cytokine Staining (ICS)

To 1 ml of 2 x 10^6 PBMC/mL in complete RPMI media (in 17x100mm round bottom polypropylene tubes (Sarstedt, Newton, NC)), anti-hCD28 (clone L293, Becton-Dickinson) and anti-hCD49d (clone L25, Becton-Dickinson) monoclonal antibodies were added to a final concentration of 1 μg/mL. For gag-specific stimulation, 10 μL of the peptide pool (at 0.4 mg/mL per peptide) were added. The tubes were incubated at 37 °C for 1 hr., after which 20 μL of 5 mg/mL of brefeldin A (Sigma) were added. The cells were incubated for 16 hr at 37 °C, 5% CO_2, 90% humidity. 4 mL cold PBS/2%FBS were added to each tube and the cells were pelleted for 10 min at 1200 rpm. The cells were re-suspended in PBS/2%FBS and stained (30 min, 4 °C) for surface markers using several fluorescent-tagged mAbs: 20 μL per tube anti-hCD3-APC, clone FN-18 (Biosource); 20 μL anti-hCD8-PerCP, clone SK1 (Becton Dickinson); and 20 μL anti-hCD4-PE, clone SK3 (Becton Dickinson). Sample handling from this stage was conducted in the dark. The cells were washed and incubated in 750 μL 1xFACS Perm buffer (Becton Dickinson) for 10 min at room temperature. The cells were pelleted and re-suspended in PBS/2%FBS and 0.1 μg of FITC-anti-hIFN-γ, clone MD-1 (Biosource) was added. After 30 min incubation, the cells were washed and re-suspended in PBS. Samples were analyzed using all four color channels of the Becton Dickinson FACSCalibur instrument. To analyze the data, the low side- and forward-scatter lymphocyte population was initially gated; a common fluorescence

cut-off for cytokine-positive events was used for both $CD4^+$ and $CD8^+$ populations, and for both mock and gag-peptide reaction tubes of a sample.

E. Results

Expression: Serum samples prior to and after the injection were analyzed for circulating SEAP activities and the results are shown in Figure 4. Results indicate that the peak levels of SEAP protein produced by the alternative adenovirus serotype were lower than but were within 3-fold of that of MRKAd5 at the same high dose level of 10^11 vp (Figure 4). The levels of SEAP in the serum dropped dramatically after day 10 and were close to background as early as day 15. These observations strongly indicate that the Ad34-based vector is efficient in expressing a transgene following intramuscular administration in a primate.

Immunogenicity: Vaccine-induced T cell responses against HIV-1 gag were quantified using IFN-gamma ELISPOT assay against a pool of 20-aa peptides that encompassed the entire protein sequence. The results are shown in Figure 5; they are expressed as the number of spot-forming cells (SFC) per million peripheral blood mononuclear cells (PBMCs) that responded to the peptide pool or the mock (no peptide) control.

Immunization with gag-expressing Ad34 vector induced detectable levels of circulating gag-specific T cells immediately after a single dose of the vector. The responses improved following a second dose given at wk 4. Overall, the responses to the Ad34-based vector were slightly lower than those induced by the same dose of MRKAd5-gag. The results strongly indicate the Ad34-based vector can prime effectively for HIV-specific T cell responses.

IFN-γ ICS analyses of the PBMC from the Ad34-immunized animals revealed that the vector can induce detectable levels of both $CD4^+$ and $CD8^+$ HIV-specific T cells (Figure 6).

Example 6

Heterologous Immunization

Cohorts of 3 monkeys were immunized (at wks 0, 4) with 10^11 vp Ad34ΔE1gagΔE4Ad5Orf6 followed by a booster at week 24 with 10^10 vp Ad35ΔE1gagΔE4Ad5Orf6. Vaccine-induced T cell responses against HIV-1 gag were quantified using IFN-gamma ELISPOT assay against a pool of 20-aa peptides that encompassed the entire protein sequence. The results are shown in Figure 12; they are expressed as the number of spot-forming cells (SFC) per million peripheral blood mononuclear cells (PBMCs) that responded to the peptide pool or the mock (no peptide) control.

Immunization with gag-expressing Ad34 vector induced detectable levels of circulating gag-specific T cells that decreased to between 94-139 SFC/10^6 PBMC at the time of the boost. Heterologous immunization with an Ad35-based HIV vector resulted in as much as a 3-fold increase in T cell responses.

IFN-γ ICS analyses of the PBMCs from the Ad34 primed/Ad35 boosted animals at week 28 revealed that the vector can induce detectable levels of both $CD4^+$ and $CD8^+$ HIV-specific T cells (Figure 13).